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Review

Notch Signaling: The Core Pathway and Its Posttranslational Regulation

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Notch signaling controls numerous cell-fate specification events in multicellular organisms, and dysregulated Notch signaling causes several diseases with underlying developmental defects. A key step in Notch receptor activation is its intramembrane proteolysis, which releases an intracellular fragment that participates directly in transcriptional regulation of nuclear target genes. Despite the apparent simplicity of this mechanism, a host of posttranslational processes regulate Notch activity during its synthesis and secretion, ligand-dependent activation at the surface, endocytic trafficking, and degradation. This review describes the core developmental logic of Notch signaling and how regulatory mechanisms tailor Notch pathway outputs to specific developmental scenarios.

Introduction

An enduring challenge in the field of developmental biology is to understand how multicellular tissues, organs, and whole animals form with such remarkable fidelity, and how perturbations in normal developmental processes contribute to human disease. Indeed, contemplating these issues long before the advent of modern molecular biology, an eighteenth-century commentator expressed sentiments that are still true today:

> "Considering the wonderful frame of the human body, this infinitely complicated engine, in which, to the due performance of the several functions and offices of life, so many strings and springs, so many receptacles and channels are necessary, and all to be in their right frame and order; and in which, besides the infinite, imperceptible and secret ways of mortality, there are so many sluices and flood-gates to let death in, and life out, it is next to a miracle we survived the day we were born." (Puckle, 1798)

Surprisingly, research over the past few decades has revealed that the orderly differentiation and arrangement of these many physiological "strings and springs" are controlled by a relatively small number of developmental signaling pathways. These pathways, including the Notch, Ras/MAPK, Hedgehog, Wnt, TGFβ, and JAK/STAT pathways, among others, are widely conserved throughout the animal kingdom and they cooperate throughout development to pattern a diverse array of tissues in different animal species. One of these key pathways—the Notch signaling pathway—is named after X-linked, dominant *Drosophila* genetic mutants that were first isolated sometime prior to 1916 and that exhibit irregular notches of missing tissue at the tips of the insect wing blades (Mohr, 1919; Morgan and Bridges, 1916). However, the developmental role of Notch was not appreciated until the 1930s, when complete loss of *Notch* gene activity was found to cause lethal hyperplasia of the embryonic nervous system (Poulson, 1940). During normal embryogenesis, only ~25% of ventral ectoderm cells adopt a neuroblast fate and generate the neuronal lineages, with the remaining ~75% of cells differentiating into epidermal structures. In *Notch*-deficient embryos, most of the immature ectodermal cells inappropriately select the neuroblast fate, causing a vast overproduction of neurons at the expense of the epidermis. This classical *Notch* mutant phenotype reveals a key feature of Notch signaling that applies to scores of analogous cellular patterning events in different organisms: Notch signaling often controls binary cell-fate decisions between cells that are initially equivalent with respect to their developmental potential. In *C. elegans*, for example, the Notch family member LIN-12 mediates interactions between two equivalent progenitor cells in the hermaphrodite gonad, termed the anchor cell (AC) and the ventral uterine precursor cell (VU). Normally each progenitor cell has an equal chance of adopting the AC or VU fate, but mutations that inactivate LIN-12 cause both to adopt the AC fate, whereas mutations that overactivate LIN-12 cause both to become VU cells (Greenwald et al., 1983).

Importantly, although such binary cell fate choices are classically associated with Notch signaling, the pathway is also widely used in patterning interactions that occur between cell types that are initially distinct from one another, termed inductive cell fate interactions. Examples of Notch-mediated inductive patterning events include early blastomere determinations in *C. elegans*, cone cell patterning by photoreceptor precursor cells in the fly retina, signaling across the dorsal/ventral boundary of the fly wing margin, tip-cell formation during mammalian angiogenesis, and mammalian astrocyte differentiation. During organogenesis and the formation of other complex tissues, Notch signaling can be employed in both the binary cell choice and inductive modes to generate complicated patterns of differentiated cell types. For example, in the *Drosophila* retina and mouse pancreas, Notch signaling is used reiteratively to generate a spatially intermingled pattern of cells with distinct structural and physiological roles (Cagan and Ready, 1989; Murtaugh et al., 2003). In vertebrates, Notch signaling has been implicated in many such patterning events as diverse as inner ear hair cell formation, insulin-secreting pancreatic β cell production, specification of crypt and goblet cells in the intestine, and multiple steps of B and T cell development within the immune system (Apelqvist et al., 1999; Lanford et al., 1999; Robey et al., 1996; van Es et al.,

2005; Washburn et al., 1997). Proper tissue development also requires that fine-scale cellular differentiation is coordinated with the overall control of tissue size and identity, and indeed Notch signaling is widely implicated in many fundamental regulatory processes, including cell proliferation, apoptosis, and the epithelial-mesenchymal transition in higher eukaryotes. Indeed, it might not be an exaggeration to suggest that nearly all cells of complex animal tissues could potentially require Notch signaling at some point or points in their lineage histories for proper final differentiation.

Given the wide-ranging importance of Notch signaling during animal development, it is not surprising that mutations in genes encoding Notch signaling components have been implicated in several human diseases involving aberrant cellular differentiation or tissue development, including T cell acute lymphoblastic leukemia (T-ALL), Alagille syndrome, spondylocostal dysostosis, tetralogy of Fallot, CADASIL syndrome, and aortic valve disease (reviewed in Artavanis-Tsakonas et al., 1999; Gridley, 2003). A detailed description of the clinical and molecular features of each of these syndromes is beyond the scope of this review, but it is worth noting that these diseases are all characterized by abnormal cell or tissue differentiation. For example, T-ALL involves excessive production of lymphoblasts in the immune system, Alagille syndrome includes pleiotropic developmental abnormalities affecting several tissues and organs, spondylocostal dysostosis is a disorder of vertebral development, and tetralogy of Fallot and aortic valve disease are congenital heart defects. A particularly illustrative case is provided by CADASIL syndrome, an adult-onset stroke and dementia syndrome that might suggest a role for Notch in neurological function, but which is actually caused by a developmental defect in brain vasculature that confers increased susceptibility to stroke (Ruchoux et al., 1994).

As the above examples indicate, Notch signaling most typically controls the fine cellular patterning and allocation of different cell types within a tissue. Accordingly, Notch signaling depends upon direct contact between the interacting cells. Furthermore, as Notch-dependent patterning events often occur over relatively brief developmental time spans, signal transmission must be both rapid and highly responsive to downregulation and reactivation. As is discussed below, the core molecular features of Notch signaling as well as its regulation by a host of posttranslational processes allow the pathway to operate with exquisite spatiotemporal sensitivity and versatility in a wide variety of developmental contexts.

The Core Pathway of Canonical Notch Signaling: Simple and Direct at First Glance

The term ''Notch signaling'' is generally understood to refer to a specific molecular mechanism that is highly conserved among many organisms and is the best characterized mode of Notch signaling. This mechanism is also referred to as ''canonical'' Notch signaling, to distinguish it from some atypical signaling modes that have also been documented. This review generally describes canonical Notch signaling and its regulatory control, unless indicated otherwise.

Canonical Notch signaling involves activation of the Notch receptor at the cell surface by ligands of the DSL family, which includes Delta and Serrate/Jagged in *Drosophila* and mammals as well as LAG-2 in *C. elegans* (Figure 1). Members of both the Notch receptor and DSL ligand families are, for the most part, type I single-pass integral membrane proteins with extracellular domains consisting primarily of up to 36 tandem EGF-like repeats (Wharton et al., 1985; Yochem and Greenwald, 1989). Receptor-ligand interactions involve direct binding of an N-terminal ligand domain to the EGF-like repeat 11-12 region of Notch (Rebay et al., 1991). As revealed by a recent atomic force microscopy study (Ahimou et al., 2004), the binding of Delta to Notch is extremely strong compared with other receptor-ligand interactions, which presumably helps generate the physical force needed to dissociate and activate the receptor. An unusual class of secreted DSL ligands has also been defined and characterized in *C. elegans*, where they appear to cooperate with membrane-anchored DSL ligands in certain Notch/LIN-12-dependent developmental patterning events (Chen and Greenwald, 2004; Komatsu et al., 2008). As discussed below, it is thought that Notch activation depends critically upon dynamic interactions between membrane-bound Notch receptors and ligands during direct cell-cell contact and endocytosis. Thus, it is unlikely that the secreted ligands are able to activate Notch on their own; instead, they evidently pair with membrane-anchored ligands to achieve productive receptor activation, although whether the secreted and transmembrane ligands interact directly or through associations with other extracellular components remains to be determined. Phyllogenetic and molecular analyses indicate that specific subclasses of secreted and transmembrane ligands are likely to function together, and that certain mammalian DSL ligands might also participate in similar paired-ligand interactions, even though all known mammalian DSL ligands belong to the membrane-anchored class (Komatsu et al., 2008).

Studies have also identified alternative non-DSL-type Notch ligands, including the adhesion molecule F3/Contactin and the EGF-repeat factor DNER, which activate mammalian Notch during oligodendrocyte maturation and Bergmann glial cell differentiation, respectively (Eiraku et al., 2005; Hu et al., 2003). The mammalian microfibrillar proteins MAGP-1 and MAGP-2 are also capable of activating Notch, and MAGP-2 also interacts with and promotes extracellular shedding of DSL ligands, indicating that these small microfibril-associated proteins might have multiple effects on Notch signaling in different tissues (Miyamoto et al., 2006). Intriguingly, MAGP proteins only activate Notch receptors expressed in *cis* in the same cell, in contrast to the *trans* mode of ligand-induced Notch activation seen with either DSL ligands or the other atypical Notch ligands that are presented by neighboring cells. Whether these alternative ligands represent rare examples of versatile cell-surface factors that are co-opted to regulate Notch in unusual developmental contexts, and whether Notch activation by atypical ligands is much more prevalent than currently appreciated, remain open questions raised by these intriguing findings.

Ligand binding activates Notch through a process involving proteolysis and endocytosis of the receptor. These events are highly regulated and the net result is intramembrane proteolysis of the intact, membrane-bound Notch receptor and consequent release of a soluble fragment consisting of the entire intracellular domain, termed Notch intracellular domain (NICD; Figure 1; reviewed in Bray, 2006; Gordon et al., 2008). NICD possesses

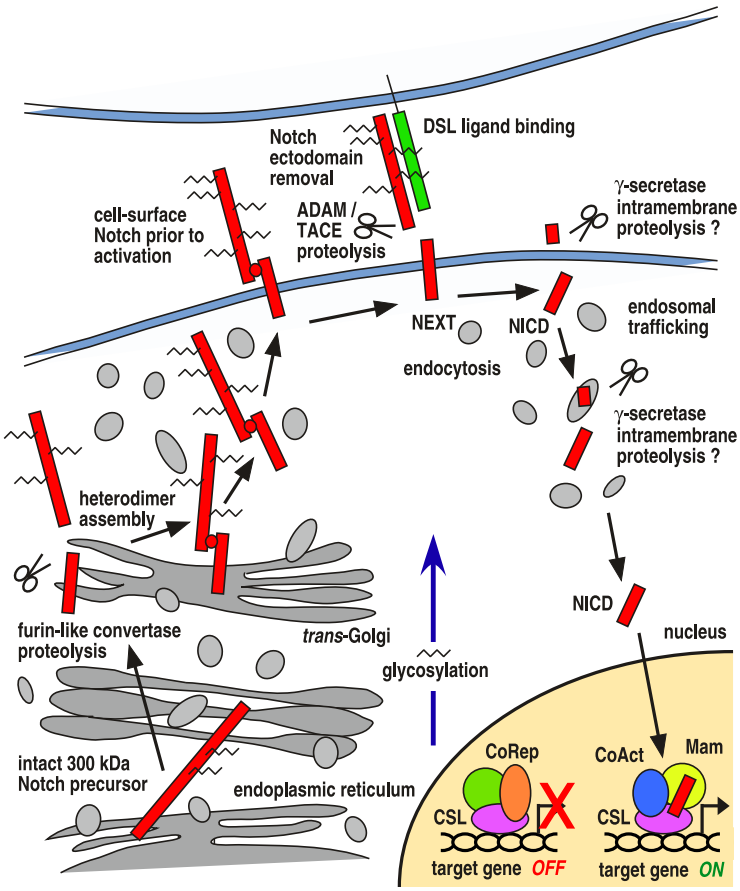

Figure 1. Overview of Notch Receptor Synthesis and Activation

The Notch receptor is synthesized as a 300 kDa precursor that is cleaved by furin-like convertase(s) in the *trans*-Golgi compartment. The resulting extracellular/lumenal N-terminal fragment and transmembrane domain/intracellular domain C-terminal fragment are assembled into the mature heterodimer receptor through a noncovalent linkage. The extracellular/lumenal portion of Notch undergoes extensive *N*- and *O*-linked glycosylation during Notch synthesis and secretion, which is critical for proper folding of the receptor and its subsequent interactions with ligands. Following export to the cell surface, Notch signal transduction is initiated by ligand binding and endocytosis, which generate the forces needed to expose an otherwise inaccessible ADAM10/TACE/Kuz/SUP-17 cleavage site in the extracellular portion of the Notch C-terminal fragment. Cleavage at this site produces the activated, membrane-anchored Notch form termed Notch extracellular truncation (NEXT). NEXT is subsequently cleaved by the intramembrane aspartyl protease complex γ-secretase, leading to release of the Notch intracellular signal-transducing fragment termed Notch intracellular domain (NICD). This cleavage can occur at the cell surface and within the endosomal trafficking pathway. In the absence of NICD, most Notch target genes are maintained in an actively repressed state through the formation of transcriptional complexes involving CSL transcription factors and various corepressors (CoRep). Upon nuclear translocation of NICD, corepressors associated with CSL are displaced and a transcriptionally active complex consisting of CSL, NICD, Mastermind (Mam), and coactivators (CoAct) assembles, leading to activation of Notch target genes. This schematic presents a simplified overview of the main conserved features of Notch synthesis and signaling; details of the biochemical mechanisms involved are omitted for the sake of clarity, the positions of the Notch diagrams are not intended to accurately depict the topology of Notch in various membrane compartments, and the glycosylation symbols and transcriptional complex diagrams are illustrative and do not imply specific glycosylation site locations or protein-protein interactions.

nuclear localization sequences (Lieber et al., 1993; Stifani et al., 1992) and enters nuclei where it participates directly in the transcriptional regulation of target genes (Struhl and Adachi, 1998). The direct translocation of an active Notch signaling fragment to the nucleus is arguably the most striking feature of Notch signaling, and sets this pathway apart from those that rely upon multiprotein phosphorylation cascades, second messengers, and other signal-relaying mechanisms. It is tempting to speculate that this very direct mode of Notch signaling evolved to fulfill the primary requirement of Notch-mediated cellular patterning—the rapid transmission of developmental cues from the cell surface to the nucleus in response to immediate cell-cell contact.

Additional mechanisms that modulate ligand activity toward Notch also exist. While DSL ligands presented by neighboring cells interact in *trans* with Notch to activate the pathway, the same ligands are also capable of interacting in *cis* with Notch in the same cell, causing inhibitory effects on Notch signaling (Heitzler and Simpson, 1993; Jacobsen et al., 1998). These inhibitory *cis* interactions could potentially take place in different membranous compartments in addition to the cell surface, and might contribute to the dynamic changes in Notch activation and repression that occur as different cells come into contact with one another during tissue development. Another inhibitory ligand mechanism is illustrated by mammalian Delta-like 1 (Dlk-1), which shares overall homology with other DSL ligands yet lacks the crucial Notch-binding domain, and which exerts antagonistic effects on Notch signaling in a heterologous *Drosophila* assay (Bray et al., 2008). DSL ligand activity is also influenced by many of the same posttranslational modifications that modify Notch receptors themselves, including proteolysis by metalloproteases and γ-secretase, glycosylation, and endocytic trafficking, as discussed below. The full range of posttranslational processes affecting Notch ligands and their functional consequences on signaling are areas that deserve more attention in the future.

Nuclear Events in Notch Signaling: The End Justifies the Means

The nuclear responses downstream of Notch activation are also sensitively modulated by various transcriptional mechanisms (reviewed in Kovall, 2008). The primary nuclear effectors of Notch signaling are transcription factors of the conserved mammalian CBF1/*Drosophila* Su(H)/*C. elegans* LAG-1 (CSL) family (Figure 1). In the absence of Notch activation, CSL proteins typically act as transcriptional repressors of Notch target genes in concert with corepressors including NCoR/SMRT, MINT/SHARP/SPEN, SKIP, CIR, Hairless, CtBP, and Groucho/TLE complexes (reviewed in Bray, 2006; Kovall, 2008). In *Drosophila*, many Notch target genes are also silenced by the histone chaperone Asf1, which is recruited to target promoters by CSL (Goodfellow et al., 2007). Evidence for CSL repressor functions has emerged from both molecular studies (Hsieh et al., 1996) and phenotypic studies that initially

demonstrated functional effects of the CSL repressor mode for certain cell specification events in *Drosophila* embryos and mechanosensory bristles (Barolo et al., 2002; Morel and Schweisguth, 2000). CSL-associated repressor complexes assemble on different Notch target promoters, with a great deal of variation in the arrangement of CSL binding sites, types of transcriptional repressor complexes, and resulting modulatory effects on gene expression (reviewed in Bray and Furriols, 2001). Nevertheless, some Notch target genes might not be subject to repression by CSL repressor complexes. In one recent study, genetic elimination of CSL itself or mutation of CSL binding sites did not cause derepression of the enhancer for the *C. elegans* Notch target gene *ref-1* (Neves et al., 2007).

Upon ligand-induced Notch activation, the released NICD fragment physically binds to CSL and, together with the coactivator Mastermind (Mam in *Drosophila* and mammals; LAG-3 in *C. elegans*), forms a transcriptionally active ternary complex (Figure 1; Petcherski and Kimble, 2000; Wu et al., 2000). Several recent structural studies have elucidated the detailed biophysical features of this ternary complex (reviewed in Barrick and Kopan, 2006; Kovall, 2008), revealing that conformational changes occur among CSL, NICD, and Mam/LAG-3 that drive the folding of unstructured protein segments and facilitate assembly of the active complex. Once formed, this active complex recruits general transcription factors including CBP/p300 and PCAF, promoting chromatin acetylation and increased expression of Notch target genes (Fryer et al., 2002; Wallberg et al., 2002). The complexity of the various repressor and activator nuclear complexes in Notch signaling is not yet fully understood. Presumably, assembly of alternative complexes at chromatin sites that are already occupied by CSL, either stably or transiently, might allow for rapid on-off bistable switching and differential modulation of the duration and/or intensity of different target gene outputs. Along with the restricted activation of Notch by direct cell-cell contact, the direct nuclear translocation of NICD and its participation in these dynamic transcriptional complexes ensure that signal transmission occurs with the high degree of spatiotemporal control demanded by intricate cellular patterning processes.

Once generated by irreversible proteolysis, the potent NICD signaling fragment can no longer be regulated by ligand binding or other cell-surface events, so it is critical for NICD turnover to be tightly controlled to prevent sustained signaling for an inappropriately long period or at an excessively high level. Disassembly of the CSL/NICD/Mam ternary complex and resulting signal attenuation is mediated by ubiquitination and proteosomal degradation of NICD by the E3 ubiquitin ligase Fbw7 (mammals)/SEL-10 (*C. elegans*), involving phosphorylation of NICD on its C-terminal PEST domain by cyclin-dependent kinase 8 (Fryer et al., 2004; Tsunematsu et al., 2004). Mutations that delete the Notch PEST domain lead to an inability to degrade NICD properly and account for some cases of T-ALL, emphasizing the physiological importance of this signal attenuation mechanism (Weng et al., 2004).

Lateral Inhibition and Transcriptional Feedback: The Molecular Logic behind Notch Signaling

An old but still central concept in Notch signaling is the ''lateral inhibition'' model, a transcriptional feedback mechanism that explains how Notch signaling can drive two initially identical progenitor cells to adopt different fates. Derived from both experimental work and theoretical modeling, the lateral inhibition model is a cornerstone of Notch biology that still serves as the starting point for interpreting many new Notch-related phenotypes. Early evidence for this mechanism came from laser ablation studies in the grasshopper embryo, where lethal ablation of an emerging neuroblast caused an adjacent cell, normally fated to remain epidermal, to instead differentiate as a substitute neuroblast (Doe and Goodman, 1985). The close resemblance of this cell-fate transformation to the *Notch* mutant embryonic phenotype immediately suggested that Notch signaling generates the lateral inhibitory signal from a presumptive neuroblast that normally prevents adjacent cells from adopting the same fate.

Molecular genetic studies in flies and worms subsequently elucidated the basic molecular mechanism of lateral inhibition (Figure 2). Initially, the two interacting cells are equivalent, each expressing comparable levels of the Notch receptor and DSL ligands, and thus possessing equivalent signal-sending and signal-receiving activities. Over time, a small stochastic difference in some aspect of signaling arises between the two cells. This initial difference is amplified by a transcriptional feedback loop wherein Notch signaling activates transcription of the *Enhancer of split* gene family, which encodes bHLH transcription factors that in turn repress *achaete-scute complex* (AS-C) genes (reviewed in Campos-Ortega, 1993). Notch and DSL ligand expression levels are themselves responsive to these changes, so an amplification mechanism ultimately drives the two cells to adopt distinct roles as either the signal-sending cell (upregulating Delta and downregulating Notch) or signal-receiving cell (upregulating Notch and downregulating Delta). In the classic examples of Notch-dependent cell-fate specifications cited above, the *Drosophila* neuroblast and *C. elegans* AC precursor are signal-sending cells, and conversely, the *Drosophila* epidermoblast and *C. elegans* VU precursor are signal-receiving cells. In addition to the effects of these transcriptional changes, lateral signaling is also reinforced by the *cis*-inhibitory effects of ligand on the pool of Notch expressed within the same cell (Figure 2). As mentioned above, in addition to lateral signaling between equivalent cells, Notch signaling is also used in many instances of inductive signaling between different cell types. Key features of the lateral inhibition mechanism can also apply to inductive Notch signaling, including transient activation of the pathway in both cells during the initial stages of the interaction, followed by transcriptional changes in the expression of Notch, its ligands, and target genes that refine the unilateral signaling between the distinct cell types.

In its purest form, the lateral inhibition model posits that a random ''salt-and-pepper'' pattern of differentiated cell types can emerge from an undifferentiated field of equivalent cells due to the amplification of small stochastic differences that initially arise among the interacting cells. A slight propensity of one cell to express more Notch than its neighbor, for example, could be amplified over time such that this cell adopts the signal-receiving role, with the neighboring cell thus adopting the signal-sending role. Despite years of research on Notch signaling, the nature of these small initial differences in signaling remains obscure, and indeed there might not be a common

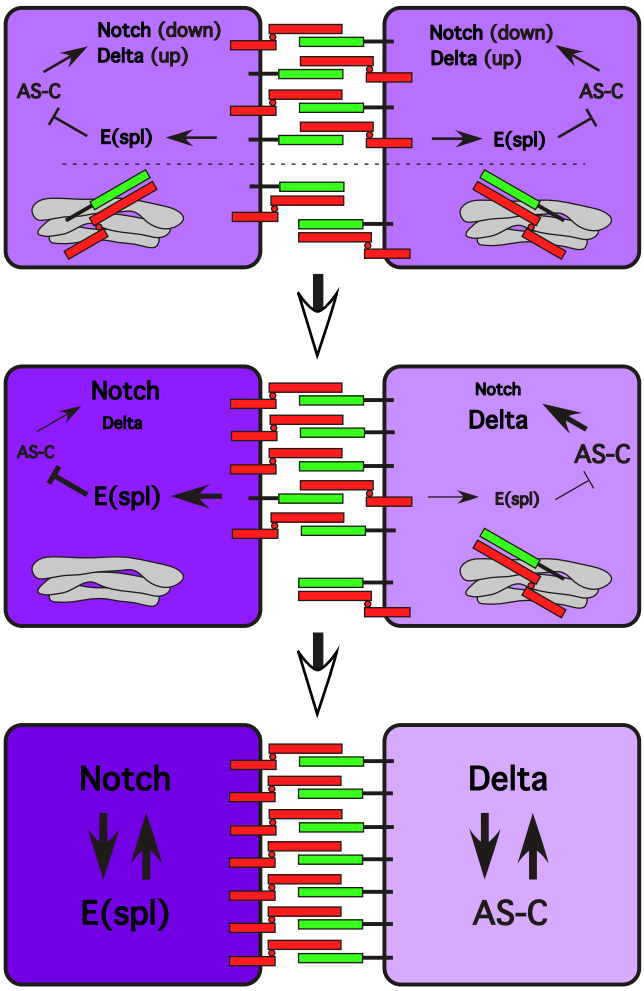

Figure 2. The Feedback Loop of Transcriptional Amplification in Notch-Mediated Lateral Inhibition

Some cell-fate specifications that depend upon Notch signaling occur between cells that are initially equivalent. A model termed "lateral inhibition" explains how Notch signaling is coupled to a transcriptional feedback mechanism, leading to different cell-fate outcomes for the initially equivalent cells. (Top) Two initially equivalent cells are depicted; both express similar amounts of Notch receptor (red) and ligand (green) at the cell surface, and thus have equivalent expression of Notch target genes of the *E(spl)/HES* family of bHLH regulators. E(spl)/HES proteins antagonize activity of the *achaete-scute* complex (AS-C) genes, which exert differential effects on expression of Notch and its ligands. In addition to the Notch activation in *trans* by ligand expressed by neighboring cells, Notch is negatively modulated by interactions with ligand in *cis* within the cell. These receptor-ligand interactions in *cis* might occur at the cell surface or within the secretory pathway, as depicted below the dashed line. (Middle) A small difference in Notch signaling activity has arisen between the interacting cells, perhaps through a stochastic event. In the cell with stronger Notch signaling, *E(spl)/HES* gene expression is elevated, inhibiting AS-C and reinforcing Notch signaling while relieving the *cis*-inhibition of Notch by ligand within the same cell (left). Conversely, in the cell with weaker Notch signaling, *E(spl)/HES* expression is downregulated and AS-C activity is reinforced, so that Notch activity is reduced while ligand expression increases (right). Increased ligand expression in the signal-sending cell acts in *trans* to amplify Notch signaling in the signal-receiving cell, establishing a stable feedback amplification loop that drives one cell to adopt the signal-sending role and the other cell to adopt the signal-receiving fate. (Bottom) The final outcome of this process is shown, in which the signal-receiving cell maintains strong receptor Notch expression and signaling through E(spl)/HES-dependent transcriptional feedback (left) while the signal-sending cell maintains strong ligand expression and repressed Notch signaling through AS-C-dependent transcriptional feedback (right). The lateral inhibition model as depicted here is a simple framework for understanding many Notch-mediated cell-fate specification events, and additional cell biological processes and specific regulatory mechanisms play important modulatory roles in this feedback mechanism in actual biological contexts.

triggering event. Transcriptional fluctuations in the levels of Notch and/or DSL ligands do not generally correlate spatially or temporally with cell-fate commitment for several key LIN-12-dependent cell specifications that were analyzed in detail in *C. elegans* (Wilkinson and Greenwald, 1995). Furthermore, the actual locations at which certain cells arise and differentiate are nonrandom for many Notch-mediated patterning events, including those that specify neuroblasts and sensory organs of the *Drosophila* nervous system (Hartenstein and Campos-Ortega, 1986; Simpson et al., 1999). It is now clear that in many developmental scenarios, other positional cues and prepatterns act in concert with lateral inhibition to coordinate the spatial allocation of different cell types. As discussed below, regulatory mechanisms have also been uncovered that exert biased effects on Notch signaling, allowing more complex, organized patterns of Notch activation than the purely random salt-and-pepper pattern of interspersed cell types.

Regulating Notch Signaling: An Irrational Exuberance of Mechanisms for Achieving Rational Outcomes

Despite the apparent simplicity of canonical Notch signaling, a seemingly bewildering array of posttranslational and cell biological processes have been uncovered in recent years that regulate different aspects of pathway function. Notch, and in many cases, its DSL ligands, is subject to proteolysis, glycosylation, ubiquitination, and other modifications such as phosphorylation. Modifications that alter the sensitivity of ligand interactions with Notch, trafficking of the receptor and its ligands through endocytic cell compartments, ubiquitination-dependent recycling and degradation of Notch, and the asymmetric partitioning of Notch regulatory factors between daughter cells during cell division all exert critical modulatory effects on pathway activity.

Proteolysis: Cutting to the Heart of the Matter

In canonical Notch signaling, maturation and activation of Notch are tightly controlled by a series of proteolytic cleavages in the vicinity of its transmembrane (TM) domain (Figure 1). Notch is synthesized as an ~300 kDa precursor protein, and cleavage in the extracellular/lumenal domain a short distance from the TM domain generates N- and C-terminal fragments (NTF and CTF). This cleavage is performed by furin-like convertases and the resulting NTF and CTF are joined by a noncovalent linkage to create the mature Notch heterodimer (Blaumueller et al., 1997; Logeat et al., 1998; Rand et al., 2000). In addition, studies in both *Drosophila* and mammalian cells have provided evidence for a pool of intact Notch at the cell surface that does not undergo furin-like cleavage and yet possesses signaling activity (Bush et al., 2001; Kidd and Lieber, 2002). Notch is usually activated at the cell surface by DSL ligand binding, which is generally believed to trigger a second proteolytic cleavage in the extracellular region of the Notch CTF at a site that only becomes exposed by ligand-induced conformational changes (Figure 1). This second cleavage step is mediated by metalloproteases of the

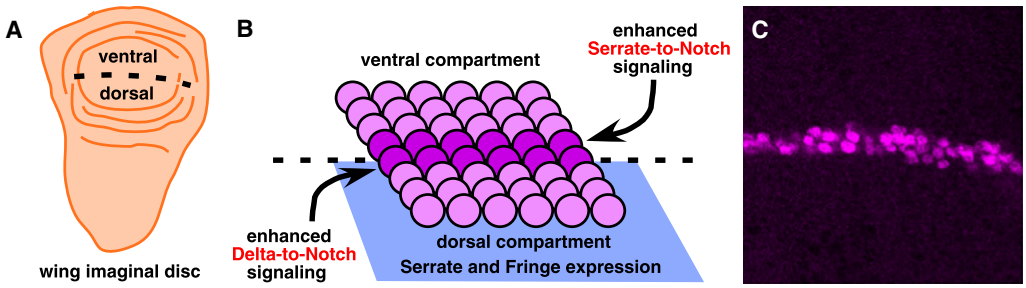

Figure 3. Modulation of Notch Signaling by Fringe-Mediated Glycosylation

The Fringe protein extends *O*-fucose chains on the Notch receptor, differentially influencing the response of Notch to its two ligands Delta and Serrate during *Drosophila* wing development.

(A) In the larval wing imaginal disc, the anterior wing margin (dashed line) is specified at the border between the dorsal and ventral compartments.

(B) Expanded view of the dorsal/ventral boundary, showing that Serrate and Fringe are expressed specifically in the dorsal compartment. Because Fringe renders Notch more sensitive to Delta and less sensitive to Serrate, dorsal cells that border the ventral compartment experience enhanced Delta-to-Notch signaling from the neighboring ventral cells expressing Delta. Conversely, ventral cells that border the dorsal compartment experience enhanced Serrate-to-Notch signaling from neighboring dorsal cells. Over time, these Fringe-dependent differences in Notch signaling become amplified in the cells lying along either side of the border and become reinforced by changes in ligand expression, creating a stripe of strong Notch activation along the dorsal/ventral border (deep purple cells).

(C) Expression of the Notch target gene *cut* along the dorsal/ventral border of a *Drosophila* larval wing imaginal disc, illustrating this stripe of elevated Notch activation (image adapted from Kanwar and Fortini, 2008).

mammalian ADAM10/TACE/*Drosophila* Kuz/*C. elegans* SUP-17 family, and it facilitates removal of the Notch ectodomain (Brou et al., 2000; Lieber et al., 2002; Mumm et al., 2000).

Ectodomain removal results in a membrane-anchored Notch CTF termed Notch extracellular truncation (NEXT) that is subsequently cleaved within the TM domain by the intramembrane aspartyl protease γ-secretase (De Strooper et al., 1999; Struhl and Greenwald, 1999). This multisubunit proteolytic complex is active in several membrane compartments of the cell and is responsible for the intramembrane cleavage of many type I integral membrane proteins (reviewed in Wolfe and Kopan, 2004). In the case of the Notch receptor, γ-secretase-mediated proteolysis generates NICD and is the final step in the elaborate three-stage proteolytic sequence, ensuring that this potent signaling fragment is only generated from Notch that has been properly synthesized, exported to the cell surface, and activated by an appropriate ligand.

Intriguingly, among the known γ-secretase substrates are several mammalian and invertebrate DSL ligands, although the physiological significance of DSL ligand cleavage in Notch signaling is still rather mysterious. It has been suggested that intramembrane proteolysis of DSL ligands, perhaps in concert with metalloprotease-induced extracellular cleavage events, contributes to ligand downregulation during lateral signaling or similar competitive signaling scenarios, or generates a soluble intracellular fragment from the ligand that translocates to the nucleus and performs a separate signaling function (Bland et al., 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003). Further complicating matters, in *Drosophila* neuroblasts and ganglion mother cells, intramembrane proteolysis of the ligand Delta seems to involve an aspartyl protease distinct from γ-secretase (Delwig et al., 2006).

Yet another form of Notch proteolysis might play an important modulatory role in the lateral inhibition feedback mechanism. Notch derivatives have been characterized in which proteolysis generates extreme C-terminal truncations of the Notch intracellular domain (Wesley and Saez, 2000). These forms notably lack the PEST domain that confers a rapid turnover rate to NICD, and hence are predicted to produce an especially stable NICD variant. Although further functional analyses of these C-terminally truncated forms remain to be performed, they might enhance lateral inhibition by ensuring that once a cell begins to produce more NICD and adopt the signal-receiving role, NICD itself is stabilized and the ongoing cell-fate acquisition process is thereby reinforced.

Glycosylation: Implications for Notch Folding and Function

The extracellular domains of Notch and its DSL ligands contain numerous potential sites for *N*-linked and *O*-linked glycosylation (reviewed in Haines and Irvine, 2003; Vodovar and Schweisguth, 2008). The effects of glycosylation on Notch signaling are complex, and almost all studies have focused on the role of *O*-linked glycosylation on the Notch receptor itself. A particularly informative and well-studied case involves the Fringe family of glycosyltransferases, which catalyze the elongation of *O*-fucose by the addition of *N*-acetylglucosamines on specific EGF-like repeats of the Notch extracellular domain (Brückner et al., 2000; Fleming et al., 1997; Ju et al., 2000; Moloney et al., 2000; Panin et al., 1997). This modification alters the responsiveness of the receptor to different DSL ligands in only certain Notch-dependent signaling processes, which do not include, for example, lateral inhibition during *Drosophila* neurogenesis. In these specific Notch signaling events, Fringe is expressed dynamically in specific patterns, leading to spatial modulation of Notch sensitivity to its different DSL ligands and an organized pattern of Notch activation within a cellular zone. Perhaps the best understood example of Fringe-dependent Notch modulation involves the upregulation of Notch in a stripe of cells separating the dorsal and ventral compartments of the *Drosophila* wing. In the presumptive wing tissue, Notch and Delta are widely expressed, but both Fringe and the ligand Serrate are restricted to the dorsal compartment (Figure 3). Fringe-dependent modification of the Notch extracellular domain renders Notch more sensitive to Delta and less sensitive to Serrate in the dorsal compartment, leading to elevated Notch activation in the stripe

of dorsal cells running along the dorsal/ventral border. Conversely, the adjacent stripe of ventral cells abutting the other side of the dorsal/ventral border also undergoes increased Notch activation, due to its lack of Fringe and relatively higher responsiveness to Serrate expressed by the neighboring dorsal cells. As a result, Notch activation is enhanced along two adjacent rows of cells that precisely abut the dorsal/ventral boundary (Figure 3). Thus, glycosylation-dependent receptor modulation causes Notch activation and its subsequent amplification to be tailored into a narrow stripe along the presumptive wing margin, a critical patterning event in wing morphogenesis. This mechanism depends at least partially on direct carbohydrate modification of Notch ligand-binding properties. Fringe extends an *O*-fucose on the critical ligand-binding EGF-like repeat 12 of Notch, and interfering with *O*-fucosylation or adding a single *N*-acetylglucosamine moiety at this EGF-like repeat has significant effects on ligand binding and Notch activation (Lei et al., 2003; Shao et al., 2003; Xu et al., 2007).

Mammalian Fringe proteins, termed Lunatic, Radical, and Manic Fringe, are likewise expressed in restricted patterns and modulate Notch signaling in analogous patterning events, such as apical epidermal ridge formation (reviewed in Haines and Irvine, 2003). However, some unexpected effects and conflicting data have emerged from in vitro and in vivo studies on these proteins. Lunatic Fringe enhances Delta-induced Notch1 activity and inhibits Jagged-induced Notch1 signaling in C2C12 myoblasts and NIH 3T3 cells (Hicks et al., 2000), consistent with the known modulatory effects of Fringe in *Drosophila*. In contrast, expression of Lunatic Fringe appears to inhibit Delta-mediated Notch1 signaling during chick somitogenesis (Dale et al., 2003); Lunatic Fringe is induced by the Mesp2 transcription factor to suppress Notch activity at segmental borders, helping to establish the oscillating ''clock-and-wavefront'' mechanism that drives somitogenesis (Morimoto et al., 2005). Further studies are warranted to elucidate the complex molecular effects and genetic regulatory hierarchies whereby Fringe glycosyltransferases influence tissue patterning during mammalian development.

A second glycosylation enzyme, the *O*-fucosyltransferase O-fut1 in *Drosophila* (Pofut1 in mammals) plays a variety of important roles in Notch signaling. O-fut1/Pofut1 adds fucose to the EGF-like extracellular repeat region of Notch, and this fucose moiety can be further extended by Fringe. Loss of O-fut1/Pofut1 causes strong *Notch*-like mutant phenotypes in flies and mice, suggesting that this enzyme is generally required for some aspect of Notch signaling (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003). Naturally, these findings initially led to the assumption that the Fringe-dependent and Fringe-independent activities of O-fut1/Pofut1 in Notch signaling are due to glycosylation of the Notch extracellular domain by the O-fut1/Pofut1 enzymatic fucosyltransferase activity. However, subsequent studies revealed that while Fringe-dependent and some Fringe-independent Notch signaling depend on direct *O*-fucosylation of Notch by O-fut1/Pofut1 (Ge and Stanley, 2008; Stahl et al., 2008), other effects of O-fut1/Pofut1 do not seem to require its fucosylation activity. O-fut1/Pofut1 evidently possesses an endoplasmic reticulum (ER) chaperone activity distinct from its *O*-fucosyltransferase activity, and in the absence of this chaperone activity, the folding and/or export of Notch to the cell surface is compromised, with commensurate effects on Notch signaling (Okajima et al., 2005; Stahl et al., 2008). In addition, *Drosophila* O-fut1 has recently been shown to promote Notch endocytosis and turnover by associating with the Notch extracellular domain at the cell surface, in yet another function that is independent of its *O*-fucosyltransferase activity (Sasamura et al., 2007). Although the fucosyltransferase activity of O-fut1 can modulate the ability of Notch to interact with its ligands at the cell surface (Okajima et al., 2003), the ability of Notch to reach the cell surface, interact with its ligands, and become activated does not seem to absolutely require the fucosyltransferase enzymatic function of O-fut1 (Okajima et al., 2005). Similarly, in mammalian cells, Pofut1 is required for the generation of optimally functional Notch receptors, but is not absolutely essential for Notch transport to the surface or signaling, and an unrelated ER glucosidase can substitute to some extent for Pofut1 in promoting Notch folding and function (Stahl et al., 2008).

A new player in the Notch glycosylation field, termed Rumi, has recently been identified in *Drosophila* (Acar et al., 2008). Rumi is a glucosyltransferase that catalyzes the addition of *O*-glucose to specific serine residues in the Notch extracellular domain in the ER. In contrast to some O-fut1 functions, the enzymatic glucosyltransferase activity of Rumi is needed for its Notch-associated functions. In the absence of Rumi activity, Notch is transported to the cell surface and binds normally to its ligand Delta, but it fails to be proteolytically processed, leading to severe Notch signaling defects in all tissues examined. Intriguingly, flies lacking Rumi display a pronounced temperature sensitivity with respect to their Notch signaling phenotypes, consistent with the idea that Rumi is required for proper folding of Notch in the ER, and that misfolded Notch produced in Rumi-deficient cells is unable to undergo normal ADAM10/TACE/Kuz/SUP-17 cleavage and ectodomain removal (Acar et al., 2008). It will be interesting to determine whether other modifying enzymes that are needed for proper folding of the Notch receptor or its ligands are present in the ER.

Endocytosis and Endosomal Trafficking: Notch Signaling Goes with the Flow

Although Notch signaling, like many other signal transduction pathways, is often depicted as a linear transmission of the signal from the cell surface to the nucleus, the cell biological details of this process are far more complicated. Genetic studies on *Drosophila* dynamin mutants first revealed that endocytosis is essential for productive Notch signaling (Parks et al., 2000; Seugnet et al., 1997). These studies, together with molecular analyses of the sequential Notch cleavages in mammalian cells, led to the idea that the dynamic forces of membrane invagination during endocytosis of Notch and its ligands might facilitate ligand-induced ectodomain removal and exposure of the ADAM10/TACE/Kuz/SUP-17 cleavage site (Brou et al., 2000; Mumm et al., 2000; Parks et al., 2000; Figure 4). Structural studies have now provided dramatic evidence in support of this model, revealing that the extracellular cleavage site is deeply embedded within Notch in the absence of ligand, and rendered accessible to cleavage by conformational changes induced by ligand binding (Gordon et al., 2007). This ''lift-and-cut'' mechanism effectively serves to prevent inappropriate Notch activation

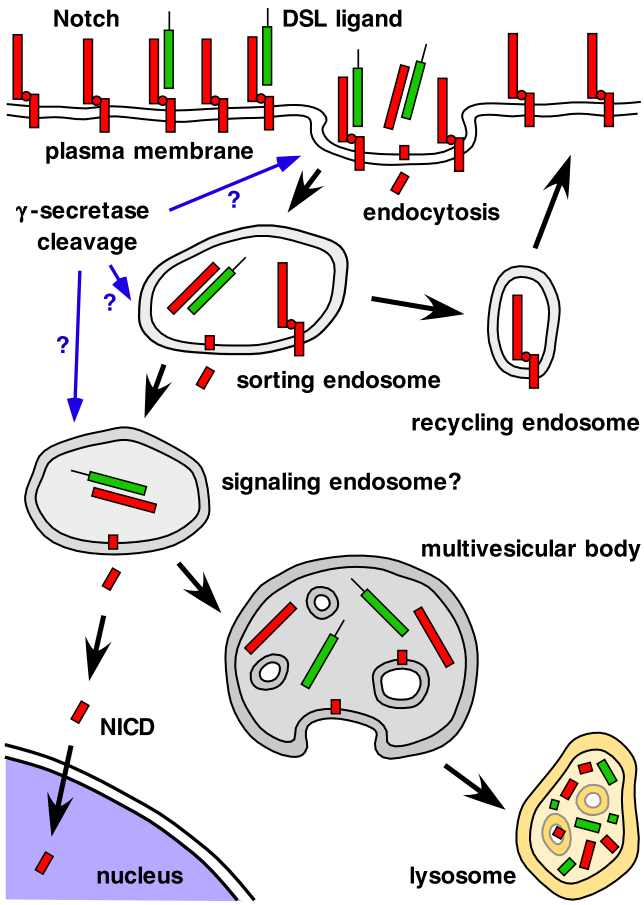

Figure 4. Overview of Notch Endocytosis and Intracellular Trafficking

At the cell surface, Notch receptors (red) interact with DSL ligands (green) presented by neighboring cells, triggering dissociation of the Notch heterodimer and exposure of the ADAM10/TACE//Kuz/SUP-17 extracellular cleavage site of the Notch C-terminal fragment (top). This process requires clathrin-dependent endocytosis of Notch in the signal-receiving cell to facilitate heterodimer dissociation and exposure of the extracellular cleavage site. Activated Notch, together with DSL ligands and nonactivated Notch, is internalized and routed into early sorting endosomes (middle). Some nonactivated intact Notch is sorted to recycling endosomes for delivery back to the cell surface, while activated Notch enters late endosomal compartments. By analogy to other activated receptors, ligand-activated Notch might be routed into a specific subpopulation of endosomes, termed signaling endosomes, with an optimal microenvironment or other properties that facilitate productive signaling (middle). Current evidence suggests that γ-secretase-mediated intramembrane proteolysis of the Notch C-terminal fragment can occur at multiple steps along this endosomal trafficking route (blue arrows). Attenuation of Notch signaling is associated with trafficking of activated Notch into late endosomal compartments, including multivesicular bodies and degradative lysosomes (bottom).

in the absence of ligand stimulation, and couples the key proteolytic steps of receptor activation to membrane trafficking processes occurring at the surfaces of interacting cells. The extracellular region of Notch responsible for shielding the cleavage site, termed the LIN-12/Notch repeats (LNR), has long been known to exert inhibitory effects on Notch signaling in *C. elegans* (Greenwald and Seydoux, 1990) and has more recently been found to play an analogous role for mouse and human Notch (Sanchez-Irizarry et al., 2004). Moreover, a recent study in *Drosophila* has shown that proper cysteine bridge formation in the LNR domain requires activity of the Ero1L thiol oxidase, and that this modification is essential for Notch folding and export to the cell surface, again emphasizing the importance of regulating ADAM10/TACE/Kuz/SUP-17 cleavage site accessibility to prevent inappropriate Notch activation (Tien et al., 2008). It should be noted, however, that some evidence suggests that ectodomain removal in the absence of this metalloprotease cleavage might suffice for some Notch activation events. For instance, *cis* activation of Notch1 by MAGP-1 and MAGP-2 is dependent upon the furin-like cleavage of Notch1 and receptor heterodimer formation, but does not require the ADAM metalloprotease cleavage (Miyamoto et al., 2006).

Interestingly, and perhaps counterintuitively, endocytosis of DSL ligands within the signal-sending cell is also necessary for Notch signaling in *Drosophila* and mammals, although apparently not in *C. elegans*. Blocking dynamin-dependent endocytosis in flies has nonautonomous as well as autonomous effects on Notch signaling, implying a requirement for endocytosis in the signal-sending cells that express the ligand Delta (Seugnet et al., 1997). Moreover, DSL ligands are detected in intracellular vesicles in *Drosophila* and mammalian cells (Itoh et al., 2003; Kooh et al., 1993). A specialized endocytic pathway, distinct from bulk endocytosis and mediated by the epsin Liquid Facets and the E3 ubiquitin ligases Neuralized and Mindbomb, potentiates ligand activity (Itoh et al., 2003; Overstreet et al., 2004; Wang and Struhl, 2004). Ligand endocytosis might contribute to Notch activation through several possible mechanisms (reviewed in Le Borgne et al., 2005). In the lift-and-cut mechanism, ligand endocytosis might be largely responsible for generating the physical force needed to pull the Notch ectodomain away from the C-terminal portion of Notch in the intact Notch heterodimer, exposing the metalloprotease cleavage site that produces NEXT and facilitating complete removal of the ectodomain. Endocytosis might also be necessary for the accumulation of DSL ligands in exosomes for subsequent delivery to the cell surface at plasma membrane concentrations sufficient for robust Notch activation. Consistent with this idea, clustering of DSL ligands can potentiate their signaling effects in mammalian cell culture assays (Hicks et al., 2002; Shimizu et al., 2002). Specific posttranslational modifications of DSL ligands in the sorting or recycling compartments, involving monoubiquitination of their intracellular domains, might also render the ligands more active (Wang and Struhl, 2004). Receptor-ligand interactions during endocytosis are further impacted by additional processes, including *trans* endocytosis of ligand into the Notch-expressing cell and the Notch ectodomain into the ligand-presenting cell (Klueg and Muskavitch, 1999), and regulation of the Neuralized E3 ubiquitin ligase by transcriptional feedback and microRNAs (Bardin and Schweisguth, 2006; Lai et al., 2005).

Upon endocytosis, Notch enters the endosomal compartment where ligand-activated Notch is sorted from non-ligand-bound Notch destined for recycling and/or degradation (Figure 4). As is the case for other signaling pathways, endocytic internalization is important for both active Notch signaling as well as its downregulation. Productive signaling from ligand-activated Notch requires not only dynamin, but also the syntaxin Avalanche and the Rab5 GTPase (Lu and Bilder, 2005), which

regulate early endosome biogenesis. Mutations in the membrane phospholipid biosynthetic enzyme phosphocholine cytidylyltransferase also affect Notch trafficking and signaling in *Drosophila*, underscoring the importance of the membrane properties of the endocytic compartment and the general role of membrane lipid homeostasis in the optimal functioning of the Notch pathway (Weber et al., 2003). In mammalian cells, endocytosis of other activated receptors is often coupled to their monoubiquitination and recruitment into clathrin-rich endosomal membrane microdomains (Gruenberg and Stenmark, 2004). An analogous process might operate in the Notch pathway, as endocytosis of Notch and its C-terminal NEXT product, as well as the subsequent cleavage by γ-secretase, has been reported to require monoubiquitination of a juxtamembrane lysine residue located near the inner face of the plasma membrane (Gupta-Rossi et al., 2004). However, this model has been challenged by a recent study showing that mutagenesis of this lysine residue does not actually block Notch cleavage, but instead shifts the cleavage site position by a few amino acids, generating an extremely unstable NICD product that is difficult to detect and unable to sustain signaling (Tagami et al., 2008).

Another Notch signal-promoting ubiquitination event is mediated by Deltex, an E3 ubiquitin ligase that acts positively in Notch signaling and influences the endosome partitioning of Notch (Hori et al., 2004). Complete loss-of-function *dx* mutant flies are viable and fertile, with *Notch*-like patterning defects, suggesting that Deltex-dependent activities define an auxiliary mode of Notch signaling (Fuwa et al., 2006). Deltex physically associates with Notch and the β-arrestin Kurtz, promoting internalization of the tripartite complex and ubiquitination of Notch (Mukherjee et al., 2005). In the absence of Kurtz, Notch protein levels are elevated, implying that Kurtz is normally required for Notch degradation. Since Deltex activity generally appears to augment Notch signaling, Kurtz might act as an E3 adaptor that interacts with Deltex and influences the relative proportions of Notch that are sorted into a signaling-competent endosome route versus a degradative endosome-lysosome pathway. Further evidence for this idea has emerged from recent genetic studies on the *Drosophila* HOPS and AP-3 complexes, which act in (1) late endosome maturation and lysosomal fusion, and (2) endosomal trafficking of proteins to the limiting membrane of the lysosome, respectively. The HOPS and AP-3 complexes are needed for Deltex-dependent, ligand-independent Notch signaling, revealing that delivery of intact, non-ligand-activated Notch to the limiting membrane of the lysosome leads to its accumulation, ectodomain shedding and/or degradation, and resulting γ-secretase-mediated activation (Wilkin et al., 2008). Considering that this Deltex-mediated Notch activation occurs largely if not completely independent of ligand stimulation, this mechanism might maintain a basal level of Notch activity that potentiates signaling or dampens signaling noise in some cells.

Studies on the Big brain (Bib) aquaporin in *Drosophila* also suggest a link between Notch signaling and endosomes. Bib is a channel protein that transports monovalent cations (Yanochko and Yool, 2002), localizes to the plasma membrane and endosomes (Doherty et al., 1997), and is needed genetically for optimal Notch signaling (Doherty et al., 1997; Rao et al., 1990). Loss of Bib was recently reported to cause two potentially linked effects in *Drosophila* imaginal tissues—an arrest in endosome maturation leading to clustering of early endosomes, and reduced acidification of the endosomal trafficking route (Kanwar and Fortini, 2008). Subsequent analysis indicates that the overt defects in Notch trafficking are attributable to an unlinked mutation present on the *bib* mutant chromosome (R. Kanwar, M.E.F., S. Bray, and T. Klein, unpublished data). Reassessment of newly recombined mutant stocks supports the idea that Bib might be needed for the normal acidification of endosomes, raising the possibility that it facilitates Notch signaling via effects on endosomes, such as regulation of their lumenal microenvironment or membrane properties. Although Bib is not essential for the γ-secretase cleavage of Notch (Kanwar and Fortini, 2008), γ-secretase is more active at low pH conditions (Pasternak et al., 2003), suggesting that the progressive acidification of the endosomal trafficking system could potentially impact levels of NICD production from Notch.

As noted above, nonactivated Notch is also internalized and undergoes endosomal trafficking for either recycling to the cell surface or degradation in the lysosome (reviewed in Kanwar and Fortini, 2004). *Drosophila* mutants lacking different components of the ESCRT complex, which sort ubiquitinated membrane proteins from early endosomes into multivesicular bodies and late endosomes (Babst, 2005), for example, exhibit cell proliferation effects due to inappropriate signaling from Notch that fails to be recycled and/or degraded normally (Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2008). Loss of the fly tumor suppressor locus *lethal giant discs* (*lgd*) likewise causes overaccumulation and ectopic activation of Notch in endosomes (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). This ectopic signaling, like ligand-regulated Notch signaling, is dependent upon γ-secretase (Jaekel and Klein, 2006; Vaccari et al., 2008), indicating that the enzymatic machinery for Notch activation exists in late endocytic compartments. Collectively, these observations suggest that Notch receptors can be proteolytically activated if appropriate cleavage sites are exposed by ectodomain dissociation, partial degradation, unfolding, or other conformational changes resembling those normally restricted to ligand-activated Notch at the cell surface or in early endocytic compartments.

Sorting of Notch into this late endocytic trafficking route also involves ubiquitination. A number of E3 ubiquitin ligases, including Suppressor of deltex (*Drosophila*)/Itch (mammals), SEL-10 (*C. elegans*), and Cbl (*Drosophila* and mammals), have been identified that target nonactivated Notch for degradation (Hubbard et al., 1997; Jehn et al., 2002; Sakata et al., 2004; Wilkin et al., 2004). In many cases, loss of one or more of these ubiquitin ligases results in Notch overactivation, suggesting a functional link to the ESCRT-dependent sorting of Notch to late endosomes and lysosomes. The complex interplay between Deltex and these degradation-promoting E3 ubiquitin ligases and whether they act directly on Notch alone, on other pathway components, or through associated cofactors are issues that need to be more fully investigated. Further insight into these mechanisms will doubtless be provided by biochemical studies identifying the specific ubiquitination events, their molecular targets, and the resulting effects on protein localization and stability. So far, these studies have proven to be challenging due to a considerable amount of functional redundancy among

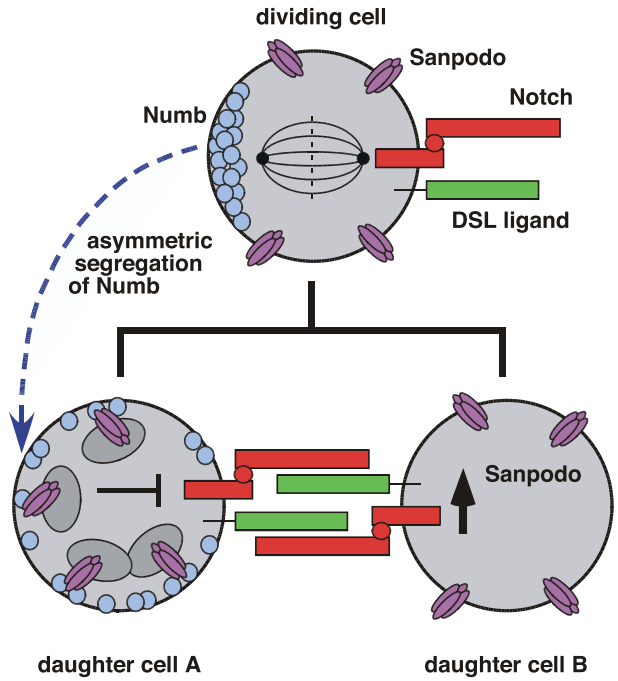

Figure 5. Modulation of Notch Signaling by Asymmetric Segregation of an Inhibitory Factor

In the mother cell prior to cell division, Numb accumulates in a crescent-shaped arc along one side of the cell, oriented toward one end of the mitotic spindle apparatus (top). During cell division, Numb segregates asymmetrically to one daughter cell (bottom left), where it inhibits Notch, either directly or by antagonizing the activity of Sanpodo, a positive modulator of Notch signaling. In the daughter cell that does not receive Numb from the mother cell, Notch activity is not inhibited to the same extent (bottom right). Asymmetric partitioning of Numb thus biases the outcome of the lateral signaling interactions between the two equipotent daughter cells, allowing the resulting cell fates to be predetermined with respect to their relative spatial orientations.

these E3 ubiquitin ligases with respect to their genetic effects on Notch signaling.

Asymmetric Segregation of Intracellular Regulators: Tipping the Balance to Achieve a Certain Outcome

In some cell lineages, cell division produces equivalent daughter cells that have an equal chance of choosing among different cell fates, as described above for the paradigmatic AC versus VU cell fate specification in *C. elegans*. However, in other instances, the Notch-mediated lateral signaling mechanism is influenced by intrinsic or extrinsic cues such that daughter cell fates are specified in a stereotyped fashion, dictated by their spatial positioning within a tissue primordium, their spatial relationship to other cells in the lineage, or their orientation relative to the mitotic cleavage plane. A fascinating example of such a mechanism involves the Numb protein, a membrane-associated phosphotyrosine-binding inhibitor of Notch that becomes localized to a crescent-shaped zone oriented with one end of the mitotic spindle in the mother cell, and subsequently segregates asymmetrically into one of the daughter cells during some cell divisions in *Drosophila* and mammalian neurogenesis (Figure 5). Numb links Notch to α-adaptin, a component of the AP2 endocytic complex that sorts cargo into transport vesicles (Berdnik et al., 2002), and some evidence suggests that this interaction targets Notch for ubiquitination and accelerated degradation in the Numb-containing daughter cell (McGill and McGlade, 2003).

An alternative explanation for the inhibitory function of Numb toward Notch is suggested by the findings that Numb still functions in the absence of its α-adaptin interaction domain (Tang et al., 2005) and that Numb also influences the subcellular localization of the Notch regulatory factor Sanpodo, a multipass transmembrane protein whose biochemical activity remains to be defined (Hutterer and Knoblich, 2005). Numb binds to Sanpodo by means of its phosphotyrosine-binding domain, and is required, along with α-adaptin, for Sanpodo endocytosis. Endocytosis of Sanpodo also depends upon the cytoskeletal factor Lethal giant larvae (Lgl) and the E3 ubiquitin ligase Neuralized, and suggests that Numb antagonizes Notch signaling indirectly by depleting the plasma-membrane-associated pool of Sanpodo, which normally enhances Notch signaling through an unknown mechanism in these asymmetric cell-fate decisions (O'Connor-Giles and Skeath, 2003; Roegiers et al., 2005).

While genetic studies indicate that Numb regulates Sanpodo endocytosis not only during mitosis but at all stages of the cell cycle (Hutterer and Knoblich, 2005), a recent study raises the intriguing idea that the asymmetric activation of Notch is restricted to actively dividing cells by virtue of their need to undergo Golgi fragmentation (Zhou et al., 2007). The Golgi protein ACBD3 is reported to associate with Numb and potentiate its inhibitory activity toward Notch, although the biochemical role of ACBD3 in these interactions is not yet understood. In quiescent cells, ACBD3 is sequestered in the Golgi and Numb remains inactive, whereas Golgi fragmentation during mitosis causes ACBD3 to be released and associate with Numb, thus coupling the asymmetric inhibition of Notch activity in one daughter cell to the mitotic process through which the daughter cells are generated.

Asymmetric segregation mechanisms also operate on DSL ligands, illustrating that a cellular strategy that directly biases Notch receptor activation can be still further exploited by applying it to the Notch ligands. Like Numb, the E3 ubiquitin ligase Neuralized is also asymmetrically partitioned among daughter cells of the *Drosophila* sensory organ precursors, and because Neuralized promotes Delta endocytosis and enhances its ability to activate Notch, this mechanism reinforces the unequal priming of Notch signaling between the two daughter cells (Le Borgne and Schweisguth, 2003). In addition, a Numb- and Neuralized-independent mechanism exists that further contributes to unequal Notch activation within the *Drosophila* sensory organ lineages. In one daughter cell, Delta activity is enhanced by its preferential entry into the Rab11-positive recycling compartment, a process that depends upon the exocyst component Sec15 (Emery et al., 2005; Jafar-Nejad et al., 2005). Conversely, in the other daughter cell, absence of the Rab11 binding partner Nuclear fallout results in an absence of recycling endosomes and loss of Delta activity (Emery et al., 2005). These different asymmetric partitioning mechanisms affecting both Notch itself and its DSL ligands reveal how the dynamic processes of intracellular protein trafficking offer many opportunities for cell biological modulation of this signaling pathway.

Remarkably, in addition to these directional signaling mechanisms involving asymmetric endocytosis of Delta and Sanpodo,

a new study reveals that asymmetric segregation of Notch- and Delta-containing endosomes that are already present in the parental cell prior to cell division also contributes to the Notch signaling bias between daughter cells (Coumailleau et al., 2009). In the *Drosophila* sensory organ precursor cell that is to undergo mitosis, specialized endosomes are found that contain Notch, Delta, and the protein Sara, and they segregate into one daughter cell in preference to the other. These Sara-positive endosomes contain γ-secretase activity and generate the active Notch signaling fragment NICD, and hence they confer an increased level of Notch signaling to the daughter cell that inherits them. This mechanism is a striking example of selective transmission of not just a single protein or asymmetric determinant, but of an intact endosomal organelle from a parental cell to one of two daughter cells. The asymmetric sorting of these Sara endosomes illustrates the great potential for exploiting the flexibility of the endosomal trafficking system in the fine-tuning of Notch signaling.

Conclusions and Future Prospects

Over the past two decades, great progress has been made in elucidating the core mechanism of the canonical Notch signaling pathway, leading to an appreciation of the central role of receptor proteolysis in this pathway. There is now a general consensus that an evolutionarily conserved series of three proteolytic steps controls assembly of the heterodimeric Notch receptor in the secretory pathway as well as receptor activation at the cell surface and in early endocytic compartments. It is tempting to speculate that this complex proteolytic mechanism evolved as a safeguard against inappropriate Notch activation and its potential oncogenic effects, an idea supported by new biophysical studies. From a clinical perspective, Notch proteolysis has emerged as an attractive target for the development of compounds that might allow Notch activity to be controlled pharmacologically, with the hope of providing new avenues for the prevention and treatment of some cancers and other disorders.

In recent years, it has become increasingly evident that in addition to the key proteolytic steps in Notch signaling, the pathway is also highly regulated by other posttranslational modifications as well as the cell biological milieu in which it operates. Understanding these diverse processes, how they are integrated at the molecular level, and how they generate the potential for sensitive spatiotemporal regulation of Notch signaling will undoubtedly remain a major focus of the field in coming years. For example, it is clear that the endosomal trafficking, recycling, and degradation of Notch are tightly linked to ubiquitination, but the molecular details of this highly dynamic process, including the identification of physiologically relevant Notch ubiquitination sites and the consequences of their alternative usage, are not well understood. The contributions of glycosylation and fucosylation to Notch folding and Notch-ligand interactions, with their enormous potential diversity of carbohydrate modifications, is also an area that still holds many secrets. Similarly, the variety of transcriptional complexes that regulate Notch target genes in the nucleus and their functional differences are only beginning to be characterized in detail. The degree to which these mechanisms are exploited by the cell for regulatory crosstalk with other developmental signaling pathways is also an area that deserves more intensive investigation, given its importance for uncovering the overall developmental logic of cell-fate acquisition and tissue patterning. Nevertheless, the recent progress that has been made in elucidating many cell biological processes with profound regulatory effects on Notch signaling has led to a much deeper understanding of the pathway complexity, despite its relatively direct mode of signal transmission from the cell surface to the nucleus. Achieving a more complete understanding of these processes, including the cell- and tissue-specific features that allow Notch signaling to operate in such a vast array of developmental contexts, will remain a major challenge for future research.

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